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Silvia Arpicco[1,*], Carlotta Lerda[1], Elisa Dalla Pozza[2], Chiara Costanzo[2], Nicolas Tsapis[3], Barbara Stella[1], Massimo Donadelli[2], Ilaria Dando[2], Elias Fattal[3], Luigi Cattel[1], Marta Palmieri[2,*]

[*]*co-corresponding authors*

[1]Dipartimento di Scienza e Tecnologia del Farmaco, University of Torino, Italy.
[2]Department of Life and Reproduction Sciences, Biochemistry Section, University of Verona, Verona, Italy.
[3]Institut Galien Paris Sud, UMR CNRS 8612, LabEx LERMIT, University of Paris-Sud, Châtenay-Malabry, France.

*Co-corresponding authors:
Silvia Arpicco
Dept. of Science and Drug Technology, University of Torino.
Via Giuria 9, 10125 Torino, Italy
Tel: +39.011.6706668
Fax +39.011.6706663
E-mail address: silvia.arpicco@unito.it

Marta Palmieri
Dept. of Life and Reproduction Sciences, Section of Biochemistry, University of Verona.
Strada Le Grazie 8, 37134 Verona, Italy
Tel: +39.045.8027169
Fax: +39.045. 8027170
E-mail address: marta.palmieri@univr.it

ABSTRACT

The aim of this work was the preparation, characterization and preliminary evaluation of the targeting ability towards pancreatic adenocarcinoma cells of liposomes containing the gemcitabine lipophilic prodrug [4-(*N*)-lauroyl-gemcitabine, C12GEM]. Hyaluronic acid (HA) was selected as targeting agent since it is biodegradable, biocompatible, can be chemically modified and its cell surface receptor CD44 is overexpressed on various tumors.

For this purpose, conjugates between a phospholipid, the 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), and HA of two different low molecular weights 4800 Da (12 disaccharidic units) and 12000 Da (32 disaccharidic units), were prepared, characterized and introduced in the liposomes during the preparation.

Different liposomal formulations were prepared and their characteristics were analyzed: size, Z potential and TEM analyses underline a difference of the HA-liposomes from the non-HA ones. In order to better understand the HA-liposome cellular localization and to evaluate their interaction with CD44 receptor, confocal microscopy studies were performed. The results demonstrate that HA facilitates the recognition of liposomes by MiaPaCa2 cells ($CD44^+$) and that the uptake increases with increasing of the polymer molecular weight.

Finally, the cytotoxicity of the different preparations was evaluated and data show that incorporation of C12GEM increases their cytotoxic activity and that HA-liposomes inhibit cell growth more than plain liposomes.

Altogether the results demonstrate the specificity of C12GEM targeting towards CD44-overexpressing pancreatic adenocarcinoma cell line using HA as a ligand.

Keywords: hyaluronic acid, CD44, gemcitabine, liposomes, pancreatic cancer.

1.Introduction

Gemcitabine (2',2'-difluoro-2'-deoxycytidine, GEM) is an anticancer nucleoside analogue of deoxycytidine that is active against solid tumors, including colon, lung, pancreatic, breast, bladder and ovarian cancers [1-3]. In particular, GEM was approved by the Food and Drug Administration in May 1996 for use against advanced or metastatic pancreatic cancer [4].

GEM is itself a prodrug since, once inside the cell, it is metabolically-activated through phosphorylation at 5' by deoxycytidine kinase, which gives the monophosphate derivative. The drug is further phosphorylated to the di- and then to the triphosphate analogue, which is incorporated into the DNA chain during replication, inhibiting DNA synthesis and cell growth [5, 6].

However, GEM is also rapidly deaminated by deoxycytidine deaminase, which locates essentially in the blood, liver and kidneys, and rapidly degrades GEM to the chemotherapeutically-inactive uracil derivative [7-9]. Thus, when administered intravenously, GEM has a very short plasma half-life, which is a major limitation of this anticancer compound [10-12].

In the clinical practice, this limitation has been overcome through a therapeutic schedule with repeated administration regimes in order to achieve therapeutic drug levels.

Recent works have been focused on an alternative approach to increase the effectiveness of the therapy by encapsulating GEM into liposomes. The results in terms of cytotoxic activity were very promising in different *in vitro* and *in vivo* studies [13-17]. In our previous work, in order to obtain both a physical and a chemical protection of the drug, we have synthesized and incorporated into liposomes lipophilic GEM prodrugs that were obtained by linking the N^4-amino group with linear acyl chains of different length which enhanced drug incorporation and antitumoral activity [18, 19].

Active targeting to tumor tissue of drug-containing liposomes is useful to further increase their activity and specificity. The overexpression of the hyaluronic acid (HA) principal cell surface receptor, CD44, on a variety of tumors such as epithelial, ovarian, colon, stomach and acute leukemia [20] makes it a vector for targeting procedure against these tumors [21, 22].

HA is a high-molecular-weight glycosaminoglycan polymer (MW = 10^6 Da) composed of repeating units of D-glucuronic acid and *N*-acetyl-D-glucosamine linked together through alternating β-1,3 and β-1,4 glycosidic bonds [23, 24]. HA has many physiological roles that include tissue and matrix water regulation, structural and space-filling properties, lubrication and a number of macromolecular functions [25].

In the preparation of liposomes targeted to tumor cells, the choice of the HA in terms of size is a crucial aspect: both high molecular weight (HMW) HA [26] and oligomers of low molecular weight (LMW) [27] have been employed.

Here, we decided to use HA of two different LMW (4800 and 12000 Da) and to compare the targeting ability of liposomes decorated with the two kinds of polymer.

The present study describes the preparation, characterization and preliminary evaluation of the biological properties on highly CD44-expressing human pancreatic adenocarcinoma cell lines of liposomes coated with HA and encapsulating a lipophilic GEM prodrug.

To this purpose, molecular conjugates between the phospholipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine and HA were synthesized and incorporated in the liposomal preparations.

2. Material and methods

2.1. Materials and instruments

Sodium hyaluronate of MW 4800 (HA_{4800}) and 12000 (HA_{12000}) Da was purchased from Lifecore Biomedical (Chaska, MN). All the phospholipids were provided by Avanti Polar-Lipids distributed by Spectra 2000 (Rome, Italy). The cholesterol and all the other chemicals were obtained from Sigma-Aldrich (Milan, Italy). All the solvents used were of analytical grade, purchased from Carlo Erba Reagenti (Milan, Italy). The ^1H nuclear magnetic resonance (NMR) spectra were recorded on a Bruker 300 Ultrashield instrument (Karlsruhe, Germany) in a mixture of D_2O, CD_3OD, CF_3CO_2D in a volume ratio of 6:3:1 at room temperature, with Me_4Si (TMS) as internal standard. The reactions were monitored by thin-layer chromatography (TLC) on F_{245} silica gel pre-coated sheets (Merck, Milan, Italy). The 4-(*N*)-lauroyl-gemcitabine (C12GEM) was synthesized according to Immordino *et al.* [18]. Fluorescein-5-(and-6)-sulfonic acid trisodium salt and Lissamine™ rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt (rhodamine-DHPE) were provided by Invitrogen (Life Technologies, Monza, Italy).

2.2. Synthesis and characterization of hyaluronated conjugates

Hyaluronated conjugates were synthesized as described by Ruhela *et al.* [28] with minor modifications. Briefly, 0.060 mmol of HA (HA_{4800} 0.3 g; HA_{12000} 0.72 g) was quite completely dissolved in 20 ml of methanol and dimethyl sulfoxide (1:1 v/v) and stirred at 60 °C. After 30 min, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) (0.038 g, 0.060 mmol) previously

dissolved in 2 ml of methanol and chloroform (1:1 v/v) was added to the HA solution. Then, acetic acid was added and the pH was adjusted at 4.5 and the mixture was stirred for 2 h at 60 °C. Subsequently, sodium triacetoxyborohydride [$NaBH(OAc)_3$] (0.038 g, 0.180 mmol) dissolved in 2 ml of methanol and chloroform (1:1 v/v) was added dropwise. The reaction proceeded for 96 h at 60 °C under magnetic stirring (Fig.1).

The reactions were monitored by TLC using chloroform/methanol (65:30 v/v). After migration of the mobile phase, sheets were exposed to iodine vapors, solution of molybdenum blue and ninhydrin (2,2-dihydroxyindene-1,3-dione) solution (100 mg/100 ml ethanol).

After removal of the solvent under reduced pressure, the crude product was resuspended in a modest amount of water. Then, in order to remove LMW unreacted compounds, the product was purified by an overnight dialysis at 4 °C against distilled water using a Spectra/Por regenerated cellulose membrane (Spectrum, Breda, The Nederlands) with a molecular cutoff of 3,500 and then freeze-dried.

The final product was purified from unreacted DPPE and HA by automated chromatography on a RediSep Rf silica column (Teledyne Isco. Inc., Lincoln, NE) on a CombiFlash Rf 200 system (Teledyne Isco. Inc., Lincoln, NE) eluted with dichloromethane-methanol (90:10 to 30:70 v/v); TLC control chloroform-methanol (65:30 v/v). The purified compounds were freeze-dried and the yield was 0.09 g, 30% for HA_{4800}-DPPE conjugate and 0.43 g, 60% for HA_{12000}-DPPE conjugate.

^{1}H NMR (D_2O, CD_3OD, CF_3CO_2D in a volume ratio of 6:3:1, 300 MHz): δ 0.9 (6 H, terminal CH_3 of DPPE), 1.3 (56 H, methylene protons of DPPE), 1.5 (4 H, NHCH_2CH_2), 2.0 (Nac-CH_3), 3.3-4.0 (sugar ring protons), 4.4-4.6 (sugar ring protons).

2.3. Preparation of liposomes

Liposomes were prepared by thin lipid film hydration and extrusion method. Briefly, a chloroform solution of the lipid components 1,2-dipalmitoyl-phosphatidylcholine (DPPC), cholesterol (Chol) and L-α-phosphatidyl-DL-glycerol sodium salt (PG) (70:30:3 molar ratios) was evaporated and the resulting lipid film was dried under vacuum overnight.

To prepare liposomes containing C12GEM, methanol solution of the prodrug and chloroform solutions of lipids were mixed in 30% ratio (mol drug/mol lipid).

Lipid films were hydrated with a 20 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulforic acid (HEPES) buffer (pH 7.4) and the suspension was vortex mixed for 10 min and bath sonicated.

The formulations were extruded (Extruder, Lipex, Vancouver, Canada) at 60° C passing the suspension 10 times under nitrogen through 100 nm polycarbonate membrane (Costar, Corning Incorporated, NY).

To prepare hyaluronated liposomes, the same method of preparation was used. Lipid films were made up of DPPC:Chol (70:30 molar ratios) and then hydrated using solution of the different HA-DPPE conjugates (3 molar ratio) in HEPES.

For the *in vitro* studies, fluorescent labeled liposomes were prepared as described above and a 10 mM solution of fluorescein-5-(and-6)-sulfonic acid trisodium salt in HEPES buffer was used during hydration.

Liposomal preparations were purified from unentrapped compounds [C12GEM or fluorescein-5-(and-6)-sulfonic acid trisodium salt] through chromatography on Sepharose CL-4B columns, eluting with HEPES buffer. Liposomes were stored at 4 °C.

2.4. Liposomes characterization

The mean particle size and polydispersity index of the liposomes were determined at 20 °C by Quasi-elastic light scattering (QELS) using a nanosizer (Coulter® N4MD, Coulter Electronics, Inc., Hialeah, FL). The selected angle was 90°, and the measurement was taken after dilution of the liposome suspensions in MilliQ® water. Each measure was carried out in triplicate. The surface charge of liposomes was evaluated by zeta potential measurements after dilution of the suspensions in 10 mM KCl using a Zetasizer (Zeta Potential Analyzer Ver. 2.17, Brookhaven Inst. Corp., Holtsville, NY).

Phospholipid phosphorous was assessed in each liposome preparation by phosphate assay after destruction with perchloric acid [29].

The amount of C12GEM incorporated in liposomes was determined by HPLC (Merck Hitachi HPLC System, Milan, Italy). The liposomal suspension was diluted by adding water and acetonitrile (60:40) to a total volume of 0.5 ml. C12GEM was extracted by adding 4.0 ml of *tert*-butyl methyl ether and vortex-mixing the sample for 30 s. The mixture was then centrifuged for 15 min at 300 x g, after which 3 ml of the organic layer was transferred and evaporated to dryness under nitrogen stream. The residue was reconstituted with 100 µl of methanol, and 40 µl of the solution were injected into a Symmetry C18 column, 5µm (Merck, Milan, Italy) equipped with a C18 column guard (Merck, Milan, Italy). The column was eluted with methanol/water (90:10 v/v) flow rate 0.8 ml/min. Detection was by UV adsorption measurement at 248 nm. Peak heights were

recorded and processed on a CBM-10A Shimadzu interface. The drug concentration was calculated from standard curves. The assay was linear over the tested concentration range (20–1000 ng).
Liposomal preparations were analyzed for physical stability in the storage conditions evaluating at different interval times diameter, zeta potential and drug leakage. Drug leakage was determined submitting 200 µl of liposomes to purification through chromatography on Sepharose CL-4B columns, eluting with HEPES buffer, and re-analyzing for drug and phospholipid content as described above. A change in content was interpreted as an indication of liposome instability.
The percentage of HA associated to the liposomes was determined by the carbazole assay [30].

2.5. Differential Scanning Calorimetry (DSC)

DSC analysis was performed on hydrated samples using a differential scanning calorimeter (DSC 7, Perkin Elmer, Waltham, MA) equipped with the Pyris software. For DSC studies, liposomes composed by DPPC alone were prepared. About 20 mg of accurately weighted samples (unloaded liposomes, C12GEM-containing liposomes either plain or hyaluronated) were introduced into a 40 µl pan and analyzed. DSC runs were conducted from 20 °C to 90 °C at a rate of 10 °C/min under a constant nitrogen stream (40 ml/min). The main transition temperature (Tm) was determined as the onset temperature of the highest peak. Calibration was achieved using indium (Tm = 156.83 °C) and n-decan (Tm=-29.6 ºC).

2.6. Transmission Electron Microscopy (TEM)

Sample preparations were performed as follows: 5 µl of each formulation were placed on 300 mesh coated copper grids (Lacey Formvar/Carbon, Ted Pella Inc., Redding, CA) allowed to adsorb and the excess amount of liquid was removed with a filter paper. Preparations were analyzed at room temperature on a Philips CM10 electron microscope operating at an accelerating voltage of 80 kV under low electron dose.

2.7. Cell culture

PaCa44, PaCa3, Panc1, CFPAC1, PT45P1, T3M4, Suit-2, MiaPaCa2, PC1J, HPAF II and PSN1 human pancreatic adenocarcinoma cell lines and VIT1 normal primary pancreatic mesenchymal cells were grown in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, 50 µg/ml gentamicin sulfate (Gibco, Life Technologies, Milan, Italy). Cells were incubated at 37 °C with 5% CO_2.

2.8. Receptor expression analysis

Flow cytometry analyses were used to determine the presence of CD44 on the cell surface by indirect staining with a secondary phycoerythrin (PE) conjugated goat antibody (Dako Italia, Milan, Italy). Cells were washed twice with PBS, incubated 30 min in the dark at 4 °C with the CD44 primary antibody and then 30 min with the secondary antibody. Samples were analyzed by a flow cytometer (FACSCanto, Becton Dickinson, San Jose, CA). Flow cytometry data were gated using the FlowJo software (TreeStar, Ashland, OR). CD44 expression was measured by calculating the ratio between median fluorescence intensity of cells labeled with antibodies versus unlabeled cells (Relative Median Fluorescence Intensity, RMFI).

2.9. Confocal laser scanning microscopy (CLSM) study

Liposome uptake was evaluated in MiaPaCa2 and VIT1 cells. Cells were seeded in 8-chamber polystyrene vessels (1.5 x 10^4 cells/well) and grown overnight at 37 °C and 5% CO_2 in culture medium. Hyaluronated or non hyaluronated liposomes labeled with fluorescein-5-(and-6)-sulfonic acid trisodium salt were added to the cells for different incubation times, from 1 up to 24 h, at 37 °C. Cells were also pre-incubated with 100x molar excess of free HMW HA (51000 Da) for 1h. At the end of the incubation periods, each well was washed twice with PBS to remove the excess of vesicles, chambers were removed, cells fixed by using a 4% paraformaldehyde solution for 15 min, and each well was washed again with PBS. Cell membranes were labeled with a 25 µg/ml rhodamine-DHPE solution for 10 min. Then, cells were washed two additional times with PBS. Cover glasses were positioned by using an antifading mounting agent. The analysis was carried out under a confocal laser scanning microscope Leica SP5 (magnification 40x with oil immersion objective) and samples visualized using the 488 nm excitation of argon laser for fluorescein and 561 nm excitation of HeNe laser for rhodamine. Fluorescein intracellular intensity was quantified by analyzing 36 cells of three different microscopic fields for each sample, by collapsing stacks and measuring fluorescence intensity corresponding to each tested cell.

2.10. In vitro cytotoxicity assays

MiaPaCa2 and VIT1 cells were plated in 96-well cell culture plates (4 x 10^3 cells/well) and incubated overnight at 37 °C with 5% CO_2. Then, cells were treated with the following samples: GEM, C12GEM and liposomes: DPPC:Chol:PG + C12GEM (lipo C12), DPPC:Chol:HA_{4800}-DPPE + C12 GEM (lipo C12 HA_{4800}), and DPPC:Chol:HA_{12000}-DPPE + C12 GEM (lipo C12 HA_{12000}) and further incubated for 72 h. At the end of the treatments cell proliferation was evaluated by

Crystal Violet (Sigma-Aldrich, Milan, Italy) staining. Five independent experiments were performed for each assay condition. Cell viability was determined photometrically (A_{595} nm).

2.11. Statistical analysis

ANOVA (post hoc Bonferroni) analysis was performed by GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). P values <0.05 were indicated as statistically significant.

3. Results and discussion

3.1. Synthesis of hyaluronated conjugates

In order to obtain liposomes containing HA and thus potentially able to bind CD44 receptor, conjugates between LMW HA and DPPE were synthesized. HA of two different MW (4800 and 12000 Da) were used with the aim to assess changes in the targeting effect as a function of polymer MW. The conjugates were prepared by reductive amination, a chemical reaction widely used in polysaccharide conjugation methods [31], using $NaBH(OAc)_3$ as reducing agent (Fig. 1). This borohydride reagent is an attractive alternative to the toxic but extensively used sodium cyanoborohydride, since it is mild and exhibits remarkable selectivity as a reducing agent [32]. The preparation of HA-DPPE conjugates consists in two steps. In the first step the aldehydic group, generated by the opening of the sugar ring, reacts in acidic medium with the amino group of DPPE forming the unstable imine. The imine formation is a very slow reaction that constitutes a limiting step of the global reductive amination. To accelerate the reaction that takes on the order of days to perform, it was carried out at 60 °C. Then the Schiff base or imine, which is formed initially *in situ* and is unstable, is reduced in presence of $NaBH(OAc)_3$ to a secondary amine leading to the formation of the conjugate. The high reaction yield was obtained using a mixture of solvents as previously reported by Ruhela [28]: methanol and dimethylsulfoxyde for the HA, methanol and chloroform for the phospholipid and for $NaBH(OAc)_3$.

Pure HA-DPPE conjugates were obtained using CombiFlash chromatography. This technique was very useful and fast and allowed to eliminate in a single step the unreacted HA and the phospholipid without loss of product. After purification and lyophilization, the conjugates were characterized by TLC: spots were ninhydrin negative and phosphate and iodine positive. Moreover, the ^1H NMR spectra of the conjugates showed the characteristic signals of both DPPE and HA moieties.

3.2 Preparation and characterization of liposomes

The HA_{4800}-DPPE or HA_{12000}-DPPE conjugates were added at a molar ratio of 3 during hydration to a lipid film composed of DPPC and Chol (70:30 molar ratio) containing 30 mol% of C12GEM. For plain liposomes PG was used instead of HA-DPPE conjugates. The physicochemical characteristics of the different formulations are summarized in Table 1. Liposomes displayed a dimensional range from about 150 nm to 190 nm and the particle size of the HA-liposomes tended to increase as the polymer MW increased. The polydispersity index was low for all the formulations (<0.15). Liposomes showed a negative Zeta potenzial value that was lower for hyaluronated liposomes compared to plain liposomes, due to the carboxylic negative residues of HA. In particular, the negative charge increased as the polymer MW increased. Those data were useful to confirm the presence of HA on the surface of the liposomes.

The efficiency of drug incorporation in fluid vesicles is strongly affected by drug polarity. Low molecular weight, water soluble molecules, such as GEM, diffuse rapidly through liposome bilayers. Our previous studies showed that the encapsulation efficiency was improved by using lipophilic GEM prodrugs [18]. Liposomes composed of DSPG:DSPC 9:1 containing the 20 mol% of C12GEM had an optimal entrapment efficiency of about 95% [18]. In the present study, similar results were obtained despite the more complex formulation consisting of actively targeted liposomes (Table 1). Thus, liposome formulations containing 30 mol% of C12GEM showed a good entrapment efficiency and the introduction of HA-DPPE conjugates did not affect liposome drug incorporation.

Results from the carbazole assay showed that the amount of HA was similar (34 nmol HA/μmol lipid) in all the liposomal preparations (containing or not C12GEM), indicating that the presence of the drug did not affect the incorporation of HA.

Stability of liposomes was evaluated in the storage condition at 4 °C in HEPES buffer. Drug leakage was determined by removing aliquots of liposomes at various time intervals and re-evaluating drug and phospholipid content after purification. Formulations were stable in the storage conditions for more than 21 days, maintaining 80% of their initial drug content. Moreover, the mean diameter of liposomes stored at 4 °C was evaluated and results showed that the liposomal preparations were stable for at least 21 days. Over this period no appreciable size change (<10% for all the preparations) and no precipitation or liposome aggregation were observed.

3.3. Differential Scanning Calorimetry (DSC)

DSC was performed to assess the possible interactions between DPPC and C12GEM and/or HA conjugated or not. The results are reported in Table 2. The thermogram of pure DPPC presented the typical pre-transition corresponding to the conversion of the lamellar gel phase $L_{\beta'}$ into the ripple gel phase $P_{\beta'}$ and the main transition related to the passage from the $P_{\beta'}$ phase to the lamellar liquid-crystalline phase L_{α} (Supplementary information Fig. S1). The pretransition occurred at T_{onset} 37.2 °C and the main phase transition at T_{onset} 41.4 °C. Thermograms were similar for DPPC in the presence of unconjugated HA_{4800} and HA_{12000}. However, the pre-transition was weaker for both molecular weights and the main transition was slightly shifted towards lower temperature with T_{onset} 40.3 °C for the higher molecular weight HA. These results demonstrate a weak interaction of HA with DPPC headgroups as already observed for a 1000 kDa HA [33]. When conjugated HA was added to hydrated DPPC, we could observe the disappearance of the pretransition in both samples. The shape of the main transition was also changed becoming broader, a sign of insertion (Supplementary information Fig. S1). The T_{onset} was decreasing slightly for HA_{4800}-DPPE (40.9 °C) and HA_{12000}-DPPE (40.6 °C). These results suggest insertion of the conjugate within the DPPC membrane.

When C12GEM was added, the pretransition was abolished and the main transition was shifted to lower temperatures (T_{onset} 36.2 °C). This indicates a strong insertion of C12GEM within the DPPC membrane due to strong hydrophobic interactions as observed for other drugs [34-38]. This phenomenon persisted in the presence of conjugated HA of both molecular weights.

3.4. Liposomes morphology

The morphology of the different liposomal preparations was observed by TEM (Fig. 2), which is the most used microscopic approach to obtain informations about liposome-drug interaction [39]. The TEM assays indicated the coexistence of spherical unilamellar vesicles having a size between 150-190 nm in diameter that was consistent with the QELS data. In the case of empty liposomes (A, C, E), no increased electron density of internal region in comparison to background was observed, while liposomes containing C12GEM (B, D and F) revealed electron-dense areas consistent with the presence of the drug. It has already been shown that TEM is a useful technique for the characterization of modified-liposomes obtained conjugating a selective ligand to liposomes [40]. In our system, hyaluronated liposomes (C, D, E and F) revealed a particulate surface coating, which was absent in plain liposomes (A and B), and were decorated by fluffy dark structures (more visible in liposomes C and E, as indicated by the arrows), suggesting that molecules of HA were exposed on liposome surface.

3.5. Analysis of CD44 expression

The HA receptor CD44, an ubiquitous transmembrane cell surface molecule, is expressed at low levels on the surface of several normal cells and overexpressed in many cancer cells [22]. The receptor levels of a panel of pancreatic adenocarcinoma cell lines were evaluated by flow cytometry, labeling the cells with an anti-CD44 antibody, with the aim to identify the cell models to be used for further *in vitro* studies.

The results reported in Table 3 show that a certain number of cell lines had a high expression of CD44, the MiaPaCa2 cells displaying the highest expression (RMFI= 485 ± 32). On the contrary, the normal primary pancreatic mesenchymal VIT1 cells did not express detectable amount of CD44. Fig. 3 shows histograms of representative flow cytometry analyses of CD44 in MiaPaCa2 and VIT1 cells. Thus, to evaluate the cellular uptake and the cytotoxic activity of the previously prepared liposomes, MiaPaCa2 and VIT1 cells were chosen as $CD44^+$ and $CD44^-$ cells, respectively.

3.6 CLSM study

Cellular uptake and localization of fluorescent plain (lipo) or hyaluronate (lipo HA_{4800} and lipo HA_{12000}) liposomes were visualized by confocal microscopy using MiaPaCa2 ($CD44^+$) and VIT1 ($CD44^-$) cells. To determine whether the liposome association to cells was due to an intracellular uptake or to a simple membrane adsorption, cell membranes were labeled with rhodamine-DHPE, while liposomes were labeled with fluorescein-5-(and-6)-sulfonic acid. This compound is more polar than carboxyfluorescein and, once inside liposomes, is relatively well retained.

Confocal analysis of the cell monolayers at 24 h (Fig. 4) showed that HA-liposome uptake by MiaPaCa2 cells, in contrast to VIT1 cells, was higher than that observed with plain liposomes, indicating a selective uptake. An increasing intensity of green fluorescence was also observed with the increasing of the HA MW on liposomes. Moreover, the fluorescence in MiaPaCa2 cells treated with HA-liposomes was mainly distributed throughout the cytoplasm, while the nuclear area remained non-fluorescent. Both MiaPaCa2 and VIT1 cells incubated with fluorescently labeled plain liposomes displayed very weak fluorescence attributable to low non-specific uptake of the particles. MiaPaCa2 cells pre-treated with a molar excess of free HA before HA-liposome incubation showed very weak fluorescent signals compared to HA untreated cells.

To study the kinetics of liposome uptake, cells were incubated with liposomes for various intervals of time (1, 2 and 24 h). The results, reported in Table 4, demonstrated that HA facilitated the recognition of liposomes by MiaPaCa2 cells and that binding of the HA-liposomes to the cells was

rapid. Liposomes containing the HA_{12000}-DPPE conjugate (lipo HA_{12000}) displayed the highest uptake by MiaPaCa2 cells, suggesting that HA with higher MW had the highest affinity for CD44. The liposome incorporation in VIT1 cells was very low and similar for all the liposomes, although it slightly increased with the time, indicating that the absence of CD44 reduced the efficiency of the uptake.

To validate the specificity of HA-liposome uptake through the CD44 receptor, MiaPaCa2 cells were pre-incubated with a saturable amount of free HMW HA (51000 Da). The uptake of HA-liposomes was inhibited by the addition of free HA, as shown by fluorescence intensity reduction, indicating that free HA competes with HA-liposomes for the receptor binding sites. In particular, a 75 % reduction in mean fluorescence in comparison with the control was observed in cells treated with lipo HA_{12000} at 24 h (Table 4). These results demonstrate that most of the HA-liposome binding to MiaPaCa2 cells is attributable to the interaction with the cell surface CD44 receptor. These findings are similar to that observed in a previous study in which the intracellular distribution of HA-grafted liposomes was evaluated [41].

3.7. In vitro cytotoxicity

Among the different GEM prodrugs previously prepared [18], C12GEM was chosen to be encapsulated in HA-liposomes for its highest cytotoxic activity on several pancreatic adenocarcinoma cell lines (data not shown). To evaluate the antiproliferative activity of C12GEM encapsulated in liposomes either plain (lipo C12) or hyaluronated (lipo C12 HA_{4800} and lipo C12 HA_{12000}), MiaPaCa2 ($CD44^+$) and VIT1 ($CD44^-$) cell growth was determined using the Crystal Violet colorimetric assay.

The results reported in Table 5 show that C12GEM displayed a cytotoxicity at 72 h similar to that of GEM on both cell types.

The incorporation of C12GEM in liposomes increased its cytotoxic activity. In particular, lipo C12 HA_{4800} and lipo C12 HA_{12000} inhibited cell growth more than non-HA-liposomes (lipo C12). This result is in agreement with the higher cellular uptake of the HA- compared to non-HA-liposomes shown by confocal microscopy analyses on MiaPaCa2 cells, that could enhance the entry of the drug into the cells and the protection against GEM inactivation.

Moreover, lipo C12 HA_{12000} displayed a stronger effect than lipo C12 HA_{4800} indicating an influence of the polymer MW in the targeting capacity. HA-liposomes loaded with doxorubicin [27, 42] and mitomycin C [26] have been already described and shown to significantly increase the toxicity of the respective drug against $CD44^+$ cells. Surprisingly, VIT1 cells, which do not express

CD44, showed a higher sensitivity towards HA-liposomes compared to non-HA-liposomes, although at a lower extent than MiaPaCa2 cells. This result is in contrast with the data obtained with confocal microscopy. However, since the in vitro cytotoxic assays were performed at 72 h, while the kinetics of cellular uptake was carried out only up to 24 h, we may speculate that in VIT1 cells a low expression of receptors other than CD44, such as RHAMM and ICAM-1 [43, 44], able to bind HA, were responsible for the higher toxicity of HA-liposomes compared to non-HA-liposomes after long time of incubation. Indeed, unpublished results have shown that HA-liposomes and non-HA-liposomes present a similar cytotoxic activity at 24 h on VIT1 cells.

4. Conclusions

Conjugates between HA of different MW (HA_{4800} and HA_{12000}) and DPPE were prepared, characterized and introduced during the preparation of liposomes encapsulating C12GEM. The liposomes were characterized in terms of size, zeta potential and C12GEM encapsulation efficiency, DSC and TEM analysis.

The CLSM analysis and the in vitro cytotoxicity test showed higher affinity of HA_{12000} to CD44 receptor in comparison with HA_{4800}.

Taken together these results demonstrate that HA-liposomes can function as tumor–targeted carrier for GEM prodrug.

Studies are in progress to deeply investigate the HA-liposome intracellular trafficking and to evaluate their *in vivo* antitumoral activity.

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References

[1] L.W. Hertel, J.S. Kroin, J.W. Misner, J.M. Tustin, Synthesis of 2-deoxy-2,2-difluoro-D-ribose and 2-deoxy-2,2-difluoro-D-ribofuranosyl nucleosides, Journal of Organic Chemistry, 53 (1988) 2406-2409.

[2] L.W. Hertel, G.B. Boder, J.S. Kroin, S.M. Rinzel, G.A. Poore, G.C. Todd, G.B. Grindey, Cellular elimination of 2',2'-difluorodeoxycytidine 5'-triphosphate - A mechanism of self-potentiation, Cancer Research, 50 (1990) 4417-4422.

[3] W. Plunkett, P. Huang, V. Gandhi, Preclinical characteristics of gemcitabine, Anti-Cancer Drugs, 6 (1995) 7-13.

[4] V. Heinemann, Gemcitabine: Progress in the treatment of pancreatic cancer, Oncology, 60 (2001) 8-18.

[5] W. Plunkett, P. Huang, C.E. Searcy, V. Gandhi, Gemcitabine: Preclinical pharmacology and mechanisms of action, Seminars in Oncology, 23 (1996) 3-15.

[6] W. Plunkett, P. Huang, Y.Z. Xu, V. Heinemann, R. Grunewald, V. Gandhi, Gemcitabine - Metabolism, mechanisms of action, and self-potentiation, Seminars in Oncology, 22 (1995) 3-10.

[7] V. Heinemann, Y.Z. Xu, S. Chubb, A. Sen, L.W. Hertel, G.B. Grindey, W. Plunkett, Cellular elimination of 2',2'-difluorodeoxycytidine 5'-triphosphate - A mechanism of self-potentiation, Cancer Research, 52 (1992) 533-539.

[8] D.Y. Bouffard, J. Laliberte, R.L. Momparler, Kinetic-studies on 2',2'-difluorodeoxycytidine (gemcitabine) with purified human deoxycytidine kinase and cytidine deaminase, Biochemical Pharmacology, 45 (1993) 1857-1861.

[9] A. Matsuda, T. Sasaki, Antitumor activity of sugar-modified cytosine nucleosides, Cancer Science, 95 (2004) 105-111.

[10] J.L. Abbruzzese, R. Grunewald, E.A. Weeks, D. Gravel, T. Adams, B. Nowak, S. Mineishi, P. Tarassoff, W. Satterlee, M.N. Raber, W. Plunkett, A phase-I clinical, plasma, and cellular pharmacology study of gemcitabine Journal of Clinical Oncology, 9 (1991) 491-498.

[11] R. Grunewald, H. Kantarjian, M. Du, K. Faucher, P. Tarassoff, W. Plunkett, Gemcitabine in leukemia - A phase-I clinical, plasma, and cellular pharmacology study, Journal of Clinical Oncology, 10 (1992) 406-413.

[12] A.M. Storniolo, S.R.B. Allerheiligen, H.L. Pearce, Preclinical, pharmacologic, and phase I studies of gemcitabine, Seminars in Oncology, 24 (1997) S2-S7.

[13] B. Pili, L.H. Reddy, C. Bourgaux, S. Lepetre-Mouelhi, D. Desmaele, P. Couvreur, Liposomal squalenoyl-gemcitabine: formulation, characterization and anticancer activity evaluation, Nanoscale, 2 (2010) 1521-1526.

[14] D. Paolino, D. Cosco, L. Racanicchi, E. Trapasso, C. Celia, M. Iannone, E. Puxeddu, G. Costante, S. Filetti, D. Russo, M. Fresta, Gemcitabine-loaded PEGylated unilamellar liposomes vs GEMZAR (R): Biodistribution, pharmacokinetic features and in vivo antitumor activity, Journal of Controlled Release, 144 (2010) 144-150.

[15] P. Jantscheff, V. Ziroli, N. Esser, R. Graeser, J. Kluth, A. Sukolinskaya, L.A. Taylor, C. Unger, U. Massing, Anti-metastatic effects of liposomal gemcitabine in a human orthotopic LNCaP prostate cancer xenograft model, Clinical & Experimental Metastasis, 26 (2009) 981-992.

[16] I.-Y. Kim, Y.-S. Kang, D.S. Lee, H.-J. Park, E.-K. Choi, Y.-K. Oh, H.-J. Son, J.-S. Kim, Antitumor activity of EGFR targeted pH-sensitive immunoliposomes encapsulating gemcitabine in A549 xenograft nude mice, Journal of Controlled Release, 140 (2009) 55-60.

[17] P. Jantscheff, N. Esser, R. Graeser, V. Ziroli, J. Kluth, C. Unger, U. Massing, Liposomal Gemcitabine (GemLip)-Efficient Drug Against Hormone-Refractory Du145 and PC-3 Prostate Cancer Xenografts, Prostate, 69 (2009) 1151-1163.

[18] M.L. Immordino, P. Brusa, F. Rocco, S. Arpicco, M. Ceruti, L. Cattel, Preparation, characterization, cytotoxicity and pharmacokinetics of liposomes containing lipophilic gemcitabine prodrugs, Journal of Controlled Release, 100 (2004) 331-346.
[19] P. Brusa, M.L. Immordino, F. Rocco, L. Cattel, Antitumor activity and pharmacokinetics of liposomes containing lipophilic gemcitabine prodrugs, Anticancer Research, 27 (2007) 195-199.
[20] B.P. Toole, T.N. Wight, M.I. Tammi, Hyaluronan-cell interactions in cancer and vascular disease, Journal of Biological Chemistry, 277 (2002) 4593-4596.
[21] M. Zoller, CD44 - Physiological expression of distinct isoforms as evidence for organ-specific metastasis formation, J. Molec. Med.-JMM, 73 (1995) 425-438.
[22] R.J.S. Sneath, D.C. Mangham, The normal structure and function of CD44 and its role in neoplasia, Journal of Clinical Pathology-Molecular Pathology, 51 (1998) 191-200.
[23] B.F. Chong, L.M. Blank, R. McLaughlin, L.K. Nielsen, Microbial hyaluronic acid production, Applied Microbiology and Biotechnology, 66 (2005) 341-351.
[24] J.R.E. Fraser, T.C. Laurent, U.B.G. Laurent, Hyaluronan: Its nature, distribution, functions and turnover, Journal of Internal Medicine, 242 (1997) 27-33.
[25] E.A. Balazs, Therapeutic use of hyaluronan, Structural Chemistry, 20 (2009) 341-349.
[26] D. Peer, R. Margalit, Loading mitomycin C inside long circulating hyaluronan targeted nano-liposomes increases its antitumor activity in three mice tumor models, International Journal of Cancer, 108 (2004) 780-789.
[27] R.E. Eliaz, F.C. Szoka, Liposome-encapsulated doxorubicin targeted to CD44: A strategy to kill CD44-overexpressing tumor cells, Cancer Research, 61 (2001) 2592-2601.
[28] D. Ruhela, K. Riviere, F.C. Szoka, Efficient synthesis of an aldehyde functionalized hyaluronic acid and its application in the preparation of hyaluronan-lipid conjugates, Bioconjugate Chemistry, 17 (2006) 1360-1363.
[29] G.R. Bartlett, Phosphorus assay in column chromatography, The Journal of biological chemistry, 234 (1959) 466-468.
[30] T. Bitter, H.M. Muir, A modified uronic acid carbazole reaction, Analytical biochemistry, 4 (1962) 330-334.
[31] D.S. Dalpathado, H. Jiang, M.A. Kater, H. Desaire, Reductive amination of carbohydrates using NaBH(OAc)(3), Analytical and Bioanalytical Chemistry, 381 (2005) 1130-1137.
[32] C.F. Nutaitis, G.W. Gribble, Chemoselective reduction of aldehydes with tetra-n-butylammonium triacetoxyborohydride, Tetrahedron Letters, 24 (1983) 4287-4290.
[33] C.G. Gaete, N. Tsapis, L. Silva, C. Bourgaux, E. Fattal, Morphology, structure and supramolecular organization of hybrid 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine-hyaluronic acid microparticles prepared by spray drying, European Journal of Pharmaceutical Sciences, 34 (2008) 12-21.
[34] K. Lohner, Effects of small organic-molecules on phospholipid phase transitions, Chemistry and Physics of Lipids, 57 (1991) 341-362.
[35] R.A. Videira, M.C. Antunes-Madeira, V.M.C. Madeira, Biophysical perturbations induced by ethylazinphos in lipid membranes, Chemistry and Physics of Lipids, 97 (1999) 139-153.
[36] A. Kostecka-Gugala, D. Latowski, K. Strzalka, Thermotropic phase behaviour of alpha-dipalmitoylphosphatidylcholine multibilayers is influenced to various extents by carotenoids containing different structural features - evidence from differential scanning calorimetry, Biochimica Et Biophysica Acta-Biomembranes, 1609 (2003) 193-202.
[37] F. Korkmaz, F. Severcan, Effect of progesterone on DPPC membrane: Evidence for lateral phase separation and inverse action in lipid dynamics, Archives of Biochemistry and Biophysics, 440 (2005) 141-147.

[38] M. Engelke, S. Tykhonova, M. Zorn-Kruppa, H. Diehl, Tamoxifen induces changes in the lipid composition of the retinal pigment epithelium cell line D407, Pharmacology & Toxicology, 91 (2002) 13-21.
[39] I.V. Zhigaltsev, N. Maurer, K. Edwards, G. Karlsson, P.R. Cullis, Formation of drug-arylsulfonate complexes inside liposomes: A novel approach to improve drug retention, Journal of Controlled Release, 110 (2006) 378-386.
[40] S. Anabousi, M. Laue, C.M. Lehr, U. Bakowsky, C. Ehrhardt, Assessing transferrin modification of liposomes by atomic force microscopy and transmission electron microscopy, European Journal of Pharmaceutics and Biopharmaceutics, 60 (2005) 295-303.
[41] H.S.S. Qhattal, X.L. Liu, Characterization of CD44-mediated cancer cell uptake and intracellular distribution of hyaluronan-grafted liposomes, Molecular Pharmaceutics, 8 (2001) 1233-1246.
[42] D. Peer, R. Margalit, Tumor-targeted hyaluronan nanoliposomes increase the antitumor activity of liposomal doxorubicin in syngeneic and human xenograft mouse tumor models, Neoplasia, 6 (2004) 343-353.
[43] B.P. Toole, Hyaluronan in morphogenesis, Journal of Internal Medicine, 242 (1997) 35-40.
[44] P.A.G. McCourt, B. Ek, N. Forsberg, S. Gustafson, Intercellular-adhesion molecule-1 is a cell-surface receptor for hyaluronan Journal of Biological Chemistry, 269 (1994) 30081-30084.

Legend to figures

Fig. 1. Reaction scheme for the synthesis of HA-DPPE conjugates.

Fig. 2. TEM images of DPPC:Chol:PG liposome (A), DPPC:Chol:PG + C12Gem liposome (B), DPPC:Chol:HA_{4800}-DPPE liposome (C), DPPC:Chol:HA_{4800}-DPPE + C12Gem liposome (D), DPPC:Chol: HA_{12000}-DPPE liposome (E) and DPPC:Chol: HA_{12000}-DPPE + C12Gem liposome (F). Arrows indicate surface coating.

Fig. 3. Representative histograms of CD44 expression in MiaPaCa2 and VIT1 cells determined by flow cytometry. Blue lines: isotype control; red lines: anti-CD44 antibody.

Fig. 4. Representative micrographs of confocal laser scanning microscopy analysis of MiaPaCa2 and VIT1 cells treated for 24 h at 37 °C with plain (lipo) or hyaluronated liposomes (lipo HA_{4800} or lipo HA_{12000}) labelled with fluorescein, in the absence or presence of HA (51000 Da) pretreatment. Green channel shows fluorescein labeled liposomes, red channel shows cellular membranes labeled with rhodamine staining, and the yellow overlay represents cellular association of liposomes. Note that green spots in merge micrographs are located outside the cells.